Biochem. J. (1972) **130**, 997–1002
Printed in Great Britain

The Effect of Hypophysectomy and Subsequent Replacement Therapy with Sheep Prolactin or Bovine Growth Hormone on the Lactose Synthetase Activity of Rabbit Mammary Gland

By E. A. JONES and A. T. COWIE
National Institute for Research in Dairying, Shinfield, Reading RG2 9AT, U.K.

(*Received* 13 *July* 1972)

1. The effects of hypophysectomy and replacement therapy with sheep prolactin and bovine growth hormone on the lactose synthetase activity of the mammary glands of lactating rabbits were studied. 2. There was an approximately fourfold decline in the lactose synthetase activity of homogenates calculated on a DNA basis within 6–7 days of hypophysectomy. Prolactin reversed this decline but growth hormone had no effect. 3. Changes in the properties of a particulate fraction isolated from the glands indicated that a decline in the effective concentration of α-lactalbumin was one factor contributing to the decreased lactose synthetase activity after hypophysectomy. 4. As the changes in lactose output produced by hypophysectomy and prolactin therapy are much greater than the changes in total lactose synthetase activity it is concluded that the activity of this enzyme is not the main factor controlling lactose output under these conditions.

Hypophysectomy of the lactating rabbit causes a fall in milk output and an almost total cessation of lactose synthesis, both these changes being reversed by treatment with sheep prolactin (Cowie *et al.*, 1969). Under these conditions the activities of some mammary-gland enzymes also change reversibly but the magnitude of these changes is too small to account for the alterations in synthetic activity (Hartmann *et al.*, 1970). However, these previous studies did not include the measurement of the activity of lactose synthetase (UDP-galactose–glucose galactosyltransferase, EC 2.4.1.22), the enzyme which is believed to catalyse the rate-limiting step in lactose synthesis (see Kuhn, 1971), and it was decided that such measurements would be of value. The study of lactose synthetase is complicated by the fact that its activity results from the interaction of a membrane-bound galactosyltransferase with α-lactalbumin, these together forming a complex capable of catalysing the synthesis of lactose at the intracellular concentrations of the substrates, glucose and UDP-galactose (see Brew, 1970). Consequently in the present study the contributions of changes in galactosyltransferase activity and α-lactalbumin concentration to changes in lactose synthetase activity were assessed by an approach previously employed in the mouse (Jones, 1972). After hypophysectomy the responses to treatment with both sheep prolactin and bovine growth hormone were studied, as the latter hormone had been shown to affect enzyme activities while having only a small influence on milk yield (Hartmann *et al.*, 1970). The results indicate that though changes in lactose synthetase activity occur after hypophysectomy and prolactin therapy they are far too small to account for the concomitant changes in lactose output.

Experimental

Animals, surgical procedures and treatments

New Zealand White rabbits were used. Details of diet, the measurement of milk yield and the technique of hypophysectomy were as previously described (Cowie *et al.*, 1969) except that hypophysectomy was carried out under halothane (Fluothane; Imperial Chemical Industries Ltd., Macclesfield, Cheshire, U.K.) anaesthesia. During the first 24h after operation the rabbits were given 25mg of ampicillin (Penbritin; Beecham Research Laboratories, Brentford, Middx., U.K.) and several subcutaneous injections of 20ml of 5% (w/v) glucose in 0.9% NaCl. Prolactin and growth hormone were used at doses previously found effective (Hartmann *et al.*, 1970) and, commencing on the third or fourth day after hypophysectomy, twice-daily subcutaneous injections of 1mg of the hormone in 1ml of 0.9% NaCl were given for 3 days. The sheep prolactin and bovine growth hormone were highly purified preparations obtained from the Endocrinology Study Section of the National Institutes of Health, Bethesda, Md., U.S.A. The sheep prolactin (NIH-PS-6) was assayed at 24.8i.u./mg and the bovine growth hormone (NIH-GH-B9) at 0.98 USP unit/mg and contained 0.4i.u. of prolactin/mg. The rabbits were killed 14–17 days after parturition and, if hypophysectomized, 6 or 7 days after the

operation. Those animals receiving hormone treatment were killed on the day after the final injection.

Preparation of homogenates and particulate fractions

The animals were killed with a blow on the base of the skull and representative samples of mammary tissue weighing 5–10g dissected out immediately. After mincing with scissors the tissue was suspended in cold mannitol–sucrose–EDTA medium (0.25M-mannitol, 0.027M-sucrose, 1mM-EDTA, pH7.4) and squeezed in muslin to minimize the amount of milk present. This and all subsequent operations were carried out at 0–4°C. A weighed portion of the minced tissue was homogenized in approx. 4vol. of medium by using a glass homogenizer and motor-driven nylon pestle with a clearance of approx. 0.2mm, 10–20 strokes being used. As some connective tissue always remained unhomogenized the suspension was strained through muslin and the volume of the strained homogenate was noted.

A portion of the homogenate was centrifuged for 5min at $600g_{av.}$ in the 40.2 rotor of a Spinco model L2 ultracentrifuge to remove whole cells and nuclei, and the supernatant was centrifuged for 30min at $57000g_{av.}$. The resulting supernatant was discarded, the pellet resuspended in mannitol–sucrose–EDTA medium and centrifuged for 15min at $57000g_{av.}$, and the pellet from this centrifugation was resuspended by hand homogenization in medium to a known volume to give the washed particulate fraction.

Assay of lactose synthetase

This was carried out as previously described (Jones, 1972) with the modification that 2mM-UTP was present during the initial reaction. Rabbit mammary homogenates contain appreciable UDP-galactose hydrolase activity, and Coffey & Reithel (1968) have shown that UTP inhibits this reaction without interfering with the synthesis of lactose.

Analytical procedures

The lactose content of milk was assayed as previously described (Cowie *et al.*, 1969), DNA by the method of Burton (1956) as modified by Munford (1963), calf thymus DNA being used as standard, and protein by a micro-Kjeldahl procedure.

Materials

Bovine α-lactalbumin was generously provided by Dr. T. E. Barman (this Institute) and UDP-galactose by Dr. P. Andrews (this Institute). Other reagents were obtained from normal commercial sources unless otherwise stated.

Results

Changes in milk and lactose yields

The effects of hypophysectomy and replacement therapy on the yield and composition of milk have already been reported in some detail (Cowie *et al.*, 1969; Hartmann *et al.*, 1970) and were re-investigated during the current work only to the extent necessary to confirm that the expected responses were being obtained. Milk yields were measured for all the rabbits (Table 1) and declined sharply after hypophysectomy. Prolactin therapy produced a partial recovery in milk yield, but was not sufficiently prolonged to produce complete recovery, whereas growth hormone produced no response. Lactose yields were determined for only some of the groups, but this served to confirm that they decreased to very low values after hypophysectomy and that prolactin therapy produced a striking recovery towards normal values.

Intracellular distribution of lactose synthetase activity

In all tissues so far investigated, including the rat mammary gland (Keenan *et al.*, 1970), galactosyltransferase appears to occur exclusively as a com-

Table 1. *Effect of hypophysectomy and replacement therapy on milk and lactose yields*

All values are presented as the means ± S.E.M. of the yields in g/day. The milk yields are the average values for the last 2 days of the particular regimen and the lactose yields are for the last or penultimate day. X, Not assayed.

Therapy ...		None	Sheep prolactin	Bovine growth hormone
No. of rabbits ...		5	4	4
Before operation	Milk	203.9 ± 20.2	227.1 ± 27.9	219.7 ± 17.8
	Lactose	4.77 ± 0.50	4.65 ± 0.61	X
After operation;	Milk	23.3 ± 5.0	38.2 ± 1.7	38.0 ± 14.4
no therapy	Lactose	0.0052 ± 0.0021	X	X
After operation;	Milk	—	80.0 ± 6.0	29.5 ± 11.3
therapy	Lactose	—	1.98 ± 0.31	X

Table 2. *Intracellular distribution of lactose synthetase activity in a mammary-gland homogenate*

The homogenate was prepared from the mammary glands of a lactating rabbit as described in the Experimental section. Fractions P_1 and P_2 were prepared by successive centrifugation at $600g_{av.}$ for 5min and $57000g_{av.}$ for 45min in the 40.2 rotor of a Spinco model L2 ultracentrifuge. The pellets were resuspended in medium without further washing and lactose synthetase activity was assayed under standard conditions in the presence of 30μM-α-lactalbumin.

	Homogenate	P_1	P_2	Supernatant	Recovery (%)
Total activity (nmol/min)	257.2	11.2	91.9	10.0	—
Percentage of homogenate activity	100	4.3	35.7	3.9	43.9
Total protein (mg)	184	27.3	34.4	102	—
Percentage of homogenate protein	100	14.8	18.7	55.5	89

ponent of the Golgi apparatus, though in the mammary gland it is also found in a soluble form in the milk. The fractionation of a rabbit mammary-gland homogenate by differential centrifugation (Table 2) showed that a large part of the lactose synthetase activity in this species also was particle-bound, and though the nature of the active particles was not characterized in the present work they may be presumed to consist of intact or disrupted parts of the Golgi apparatus. In contrast to work with the mouse (Jones, 1972), recoveries of total homogenate activity in isolated fractions were poor, the value in Table 2 of 44% being typical; the reasons for this were not established, as recombinations of the fractions did not yield enhanced activities. Because of this low and variable (25–50%) recovery of activity in the particulate fraction the total activity of the glands had to be measured by using whole homogenates, though these have the disadvantage of containing high initial concentrations of lactose, which decrease the accuracy of the enzyme assay, and unknown concentrations of α-lactalbumin. The particulate fraction was used to study changes in the effective α-lactalbumin concentration of vesicles isolated from animals in different physiological states.

Changes in particulate lactose synthetase activity after hypophysectomy and replacement therapy

In characterizing the properties of the washed particulate fraction the general approach used previously for mouse mammary lactose synthetase was followed (Jones, 1972). This assumes that the preparation contains intact vesicles capable of synthesizing lactose without further addition of α-lactalbumin, and membrane fragments, which have an absolute requirement for α-lactalbumin. Endogenous activity is assayed in the absence of added bovine α-lactalbumin and is a function of the galactosyltransferase in the preparation, which is able to combine with rabbit α-lactalbumin retained within the vesicles. Exposed activity is obtained by subtracting the endogenous activity from activity assayed in the presence of bovine α-lactalbumin (30μM). Total lactose synthetase activity is assayed in the presence of 30μM-α-lactalbumin after the addition to the particulate preparation of an equal volume of 1% (w/v) digitonin in 0.1M-tris–HCl, pH7.4, 30min before assay. This treatment disrupts the structure of the vesicles and leaves the galactosyltransferase exposed to the added α-lactalbumin. The ratio of exposed activity to total activity (ratio *A*, Table 3) may be taken as a measure of the amount of disruption of Golgi structure that has occurred during the preparative procedure. The ratio of total minus exposed activity (i.e. the activity of the galactosyltransferase within the vesicles assayed in the presence of bovine 30μM-α-lactalbumin) to the endogenous activity (ratio *B*) is inversely related to the α-lactalbumin concentration within the vesicles. In the absence of information on the interaction of bovine and rabbit α-lactalbumin with rabbit galactosyltransferase the actual concentration of α-lactalbumin within the isolated vesicles cannot be calculated, but ratio *B* provides a means of monitoring changes in this concentration.

The results in Table 3 show that though some changes occur in particulate lactose synthetase activity they are small compared with the changes in lactose output. The endogenous activity declined after hypophysectomy in the untreated group ($P<0.05$) and the growth-hormone-treated group ($P<0.01$), but in the prolactin-treated group was not significantly different from the control values. This decrease resulted from a decrease in the specific activity of galactosyltransferase as measured by 'total activity' and a decrease in the concentration of α-lactalbumin within the vesicles as indicated by a rise in ratio *B*. If the pooled results from the untreated and growth-hormone-treated groups are compared with the pooled results from the control and prolactin-treated

Table 3. *Lactose synthetase activities of the particulate fraction*

Details of the preparation of the particulate fraction are given in the Experimental section and the significance of the different types of lactose synthetase activity is explained in the text. Activities are expressed as mean values±S.E.M., in nmol of lactose formed/min per mg of protein.

		Hypophysectomized rabbits		
	Control rabbits	None	Sheep prolactin	Bovine growth hormone
Therapy ...	—			
No. of rabbits ...	4	5	4	4
Lactose synthetase activities				
Endogenous	1.02±0.15	0.43±0.15	1.31±0.36	0.34±0.09
Exposed	0.66±0.05	0.83±0.12	1.41±0.22	0.93±0.18
Total	4.26±0.73	3.84±1.05	6.91±1.09	3.13±0.45
Exposed/total (ratio *A*)	0.143±0.029	0.258±0.051	0.207±0.016	0.300±0.50
Total-exposed/endogenous (ratio *B*)	3.80±0.85	8.33±1.72	5.15±1.57	8.32±2.99

Table 4. *Lactose synthetase activities of homogenates*

Lactose synthetase activity was assayed under standard conditions in the presence of 30μM-α-lactalbumin. Activities are given as means±S.E.M., with the number of animals in parentheses.

		Hypophysectomized rabbits		
	Control rabbits	None	Sheep prolactin	Bovine growth hormone
Therapy ...	—			
Lactose synthetase activity				
Lactose synthesized (nmol/min per g wet wt.)	78.7±10.7 (5)	13.3±3.3 (5)	60.3±12.7 (4)	21.0±5.3 (4)
Lactose synthesized (nmol/min per mg of DNA)	195.7±26.0 (5)	48.7±9.7 (3)	211.3±47.3 (4)	73.0±19.0 (4)

groups, a procedure that seems legitimate in view of the similarity between the properties of these groups, the decrease in 'total activity' is significant ($P<0.05$), as is the increase in ratio *B* ($P<0.05$). Ratio *A* increases compared with the controls in the untreated ($P<0.2$), prolactin-treated ($P<0.2$) and growth-hormone-treated ($P<0.05$) groups. These changes suggest that hypophysectomy may lead to some breakdown of the Golgi structure or render that structure more liable to damage during homogenization, and that this change has not been fully reversed by prolactin therapy.

The decrease in 'total activity' does not necessarily indicate that the specific activity of the Golgi apparatus has fallen, as the particulate fraction is highly heterogeneous, but the approximate doubling of ratio *B* does imply that the α-lactalbumin concentration in the isolated vesicles has decreased so as to halve the lactose synthetase activity of the galactosyltransferase that is present.

Changes in homogenate lactose synthetase activity after hypophysectomy and replacement therapy

Table 4 presents the lactose synthetase activities of homogenates calculated on the basis of tissue wet weight and DNA content. The activity measured is a composite one, being the sum of the endogenous activity of intact vesicles and the activity of particulate and soluble galactosyltransferase exposed to added α-lactalbumin. However, as the results obtained with the particulate preparation (Table 3) indicate that changes in endogenous α-lactalbumin concentration are not large, most of the changes in homogenate activity are probably due to alterations in the galactosyltransferase content, though other factors such as the presence of inhibitors cannot be ruled out. Activity expressed on either basis decreased significantly ($P<0.01$) after hypophysectomy and was restored to the control value by prolactin therapy. The apparent slight response to growth-hormone therapy is not significant on either basis ($P<0.4$).

Discussion

One of the aims of the work described in the present paper was to test the hypothesis that the large decrease in the rate of lactose production that follows hypophysectomy in the rabbit is a result of a fall in the activity of lactose synthetase. A necessary precondition for this is that lactose synthetase must catalyse a rate-limiting reaction on the pathway of lactose

Table 5. *Activities of enzymes concerned with lactose synthesis in the mammary gland of the lactating rabbit*

References: (1) Baldwin (1966); 35-day-post-partum albino rabbits; temp. 25°C. (2) Hartmann *et al.* (1970); 15-day-post-partum Dutch and New Zealand White rabbits; temp. 38.7°C. (3) Heitzman (1968); 11–21-day-post-partum New Zealand White rabbits; temp. 26°C. (4) This paper.

Enzyme	Activity (μmol/min per g wet wt.)	Reference
Hexokinase (EC 2.7.1.1)	0.278	(1)
Phosphoglucomutase (EC 2.7.5.1)	3.01	(1)
	6.46	(2)
UDP-glucose pyrophosphorylase (EC 2.7.7.9)	3.34	(1)
	25.0	(2)
	2.73	(3)
UDP-glucose epimerase (EC 5.1.3.2)	2.26	(1)
	1.27	(2)
Lactose synthetase (EC 2.4.1.22)	0.079	(4)

synthesis in the normally lactating rabbit. There is no direct evidence for this, but lactose synthetase does appear to be the least active enzyme of the pathway by a considerable margin. This is certainly true in the rat (see Kuhn, 1971), and some of the data in the literature collected in Table 5 suggest that the situation in the rabbit is similar, though it should be noted that the breed, stage of lactation and temperature of assay used in obtaining these data vary widely. It appears from Table 5 that hexokinase activity may also be limiting, but this enzyme was assayed on day 35 of lactation, when lactose output in the rabbit is well past its peak (Cowie, 1969). From the results in Table 4, and assuming a mammary-gland weight of 90g (Hartmann *et al.*, 1970), it can be calculated that the mean lactose synthetase activity per rabbit per day is 3.5g, which compares with an actual daily yield of 4.8g (Table 1). This calculation is subject to many possible errors. The gland weight is an average value including data for Dutch rabbits in addition to New Zealand Whites and, as discussed in detail in the Results section, the enzyme activity assayed *in vitro* is not simply related to the activity *in vivo*. However, though the value for potential lactose synthesis in the mammary gland is only a crude approximation it is evident that the ratio of potential to actual synthesis is far smaller for lactose synthetase than for phosphoglucomutase and UDP-glucose pyrophosphorylase, for which the values of this ratio are respectively 81 and 284 (Hartmann *et al.*, 1970). If a similar calculation is performed for the untreated hypophysectomized animals values of 0.22g/day for lactose synthetase activity and 0.005g/day for lactose output are obtained. Thus after hypophysectomy the ratio of enzyme activity to lactose output increases greatly, suggesting that the fall in lactose synthetase activity that occurs is not the main cause of the decline in lactose production and that even if lactose synthetase catalyses a rate-limiting step in the normally lactating rabbit it does not do so in the untreated hypophysectomized animal. This conclusion is analogous to that reached by Hartmann *et al.* (1970) for the range of enzymes they studied.

From the results obtained with particulate fractions (Table 3) and homogenates (Table 4) it can be deduced that changes in the tissue content of galactosyltransferase and the concentration of α-lactalbumin within the Golgi apparatus both contribute to the decrease in lactose synthetase activity after hypophysectomy and to its increase after prolactin treatment. Bovine growth hormone has no significant effect on either component of the enzyme. Prolactin is known to act directly on the mammary glands of pregnant or pseudopregnant rabbits to produce structural and biochemical changes associated with lactation in intact animals (Chadwick, 1962; Heitzman, 1968; Falconer & Fiddler, 1970; Fiddler *et al.*, 1971) and in cultured explants (Barnawell, 1965; Bolton & Bolton, 1970). Thus in the work reported in the present paper it can be assumed that prolactin stimulates lactose synthesis by such a direct action. However, the mechanism of this action remains obscure as none of the relevant enzyme activities that have been measured changes sufficiently to account for the increase in synthetic activity.

We thank Mrs. K. A. M. Danby, Mr. R. J. Marshall and Mr. S. C. Watson for their skilled technical assistance.

References

Baldwin, R. L. (1966) *J. Dairy Sci.* **49**, 1533–1542
Barnawell, E. B. (1965) *J. Exp. Zool.* **160**, 189–206
Bolton, C. E. & Bolton, A. E. (1970) *FEBS Lett.* **9**, 177–179

Brew, K. (1970) *Essays Biochem.* **6**, 93–119
Burton, K. (1956) *Biochem. J.* **62**, 315–323
Chadwick, A. (1962) *Biochem. J.* **85**, 554–558
Coffey, R. G. & Reithel, F. J. (1968) *Biochem. J.* **109**, 169–176
Cowie, A. T. (1969) *J. Endocrinol.* **44**, 437–450
Cowie, A. T., Hartmann, P. E. & Turvey, A. (1969) *J. Endocrinol.* **43**, 651–662
Falconer, I. R. & Fiddler, T. J. (1970) *Biochim. Biophys. Acta* **218**, 508–514
Fiddler, T. J., Birkinshaw, M. & Falconer, I. R. (1971) *J. Endocrinol.* **49**, 459–469
Hartmann, P. E., Cowie, A. T. & Hosking, Z. D. (1970) *J. Endocrinol.* **48**, 433–448
Heitzman, R. J. (1968) *J. Endocrinol.* **40**, 81–84
Jones, E. A. (1972) *Biochem. J.* **126**, 67–78
Keenan, T. W., Morre, D. J. & Cheetham, R. D. (1970) *Nature (London)* **228**, 1105–1106
Kuhn, N. J. (1971) in *Lactation* (Falconer, I. R., ed.), pp. 161–176, Butterworths, London
Munford, R. E. (1963) *J. Endocrinol.* **28**, 17–34